PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/52, 39/395, 37/02
C07K 13/00

A1

(11) International Publication Number: WO 91/16069

(43) International Publication Date: 31 October 1991 (31.10.91)

(21) International Application Number: PCT/US91/01587

(22) International Filing Date: 13 March 1991 (13.03.91)

(30) Priority data:
510,696 17 April 1990 (17.04.90) US

(71) Applicant: THE UNITED STATES OF AMERICA, as represented by THE SECRETARY, U.S. DEPARTMENT OF COMMERCE [US/US]; 5285 Port Royal Road Springfield, VA 22161 (US).

(72) Inventors: RYBAK, Susanna, M. ; 7411B Round Hill Road, Frederick, MD 21701 (US). YOULE, Richard, J. ; 10811 Keswick Street, Gartett Park, MD 20896 (US).

(74) Agents: HOLMAN, John, Clarke et al.; Fleit, Jacobson, Cohn, Price, Holman & Stern, The Jenifer Building, 400 Seventh Street, N.W., Washington, DC 20004 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: SELECTIVE CYTOTOXIC REAGENTS COMPRISING TOXIC MOIETIES DERIVED FROM MAMMALIAN PROTEINS

(57) Abstract

Pancreatic ribonuclease A coupled to human transferrin via a disulfide bond resulted in a hybrid protein that was cytotoxic to mammalian cells *in vitro*. In measuring protein synthesis capacity of K562 human erythroleukemia cells the IC_{50} for different preparations of transferrin-RNase ranged from 8 x 10^{-9} to 8 x 10^{-8} M whereas the IC_{50} of RNase or SPDP RNase alone was 1000-10,000 times higher. In a clonogenic assay 1 x 10^{-6} M of the transferrin RNase conjugate killed 6 logs of cells whereas 7 x 10^{-5} M of the SPDP modified RNase had no significant effect. The inhibition of protein synthesis by transferrin-RNase was blocked by excess transferrin or ribonuclease inhibitors in the medium demonstrating that cytotoxicity was dependent on both components of the conjugate. Human serum RNase may also exhibit receptor mediated toxicities providing a new approach to selective cell killing possibly with less systemic toxicity and, importantly, less immunogenicity than the currently employed antibody-toxin conjugates.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CS	Czechoslovakia
DE	Germany
DK	Denmark
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GN	Guinea
GR	Greece
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
PL	Poland
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

SELECTIVE CYTOTOXIC REAGENTS COMPRISING TOXIC MOIETIES DERIVED FROM MAMMALIAN PROTEINS

FIELD OF THE INVENTION

The present invention relates to directed cytotoxic reagents, including immunotoxins, that selectively kill cells having a given surface marker, consisting essentially of a toxic moiety that is derived from a mammalian protein, linked to a recognition moiety capable of specific binding with a chosen cell surface marker. In particular, the present invention relates to such cytotoxic reagents comprising mammalian proteins with ribonucleolytic activity and antibodies or receptor binding ligands that recognize tumor cells or virus-infected cells.

BACKGROUND OF THE INVENTION

Toxic enzymes from plants and bacteria such as ricin, diphtheria toxin and pseudomonas toxin have been coupled to antibodies or receptor binding ligands to generate cell-type-specific-killing reagents (Youle, R. and D. Neville, 1980, "Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin." Proc Natl Acad Sci USA 77:5483-5486; Gilliland, D., Z. Steplewski, R. Collier *et al.*, 1980, "Antibody directed cytotoxic agents: Use of monoclonal antibody to direct the action of toxin A chains to colorectal carcinoma cells." Proc Natl Acad Sci USA 77:4539-4543; Krolick, K., C. Villemez, P. Isakson *et al.*, 1980, "Selective killing of neoplastic B cells by antibodies coupled to the A chain of ricin." Proc Natl Acad Sci USA 77: 5419-5423). Notwithstanding the fact that the cell-recognition moiety is not always an antibody, these directed toxins are generally known as immunotoxins

(ITs), and will be referred to as such hereinafter. These hybrid proteins kill tumor cells, for example, which express the receptor that the antibody or ligand portion of the molecule recognizes. Under appropriate conditions, conferred by the particular receptor system, the toxin enters the cytosol, inactivates the protein synthesis machinery and causes death of the target cell. Immunotoxins are highly cytotoxic to cancer cells growing in cell culture and animal models demonstrate the potential of these reagents to treat blood borne malignancies as well as solid tumors in restricted compartments such as the intraperitoneal cavity (reviewed in Griffin, T. W. *et al.*, 1988, Immunotoxins. Boston/Dordrecht/Lancaster, Kluwer Academic Publishers, p 433; Vitetta, E.E., *et al.*, 1987, Science **238**:1098; Fitzgerald, D.J., *et al.*, 1989, J. Natl. Cancer Inst. **81**:1455). Phase 1 and 2 clinical trials have been completed or are underway and have been recently reviewed (Rybak, S.M. and Youle, R.J., 1990, "Human Cancer Immunology", in Immunology and Allergy Clinics of America, W. B. Saunders). Considerable success has been achieved in *ex vivo* treatments of bone marrow in the prevention or treatment of graft-versus-host disease. *In vivo* therapy with ITs has been disappointing, but major problems associated with IT therapy were illuminated by these initial clinical trials. Toxicity not predicted from animal studies was discovered, and the expected humoral immune responses to both the antibody and toxin portion of the hybrid molecule was found to limit the therapy to a few days. Although advances in protein design techniques promise to alleviate some of the immunogenicity associated with the antibody portion of

ITs (Bird, R. E. *et al.*, 1988, Science **242**:423; Huston, J. S., *et al.*, 1988, Proc Natl Acad Sci USA **85**:5879; Ward, E.E. *et al.*, 1989, Nature **341**:544), no solution has been forthcoming for the immunogenicity of the toxin other than immunosuppression of the patients (Khazaeli, M. *et al.*, 1988, Proceedings of AACR **29**:418). Thus, there has been a continuing need for methods and compositions that would reduce the immunogenicity of the toxic moiety of cytotoxic reagents that selectively kill cells having a given surface marker.

Human transferrin (Tfn) is a serum glycoprotein that binds and delivers iron to cells by receptor mediated endocytosis reviewed in (Youle, R. and D. Neville, 1987, Immunoconjugates: Antibody Conjugates in Radioimaging and Therapy of Cancer. Oxford, Oxford University Press). After relinquishing its iron apo-Tfn-receptor recycles to the cell surface where apo-Tfn is released to continue the cycle. Monoclonal antibodies originally isolated based upon selectivity for tumor cells have been found to react with the human transferrin receptor. Transferrin (Raso, V. and M. Basala, 1984, "A highly cytotoxic human transferrin-ricin A chain conjugate used to select receptor-modified cells." J Biol Chem. **259**:1143-1149; O'Keefe, D. and R. Draper, 1985, "Characterization of a transferrin-diphtheria toxin conjugate." J Biol Chem. **260**:932-937.) or antibodies to the Tfn-receptor (Pirker, R., D. FitzGerald, T. Hamilton *et al.*, 1985, "Anti-transferrin receptor antibody linked to pseudomonas exotoxin as a model immunotoxin in human ovarian carcinoma lines." Cancer Res. **45**: 751-757; FitzGerald, D., P. Trowbridge, I. Pastan and M. Willingham, 1983, "Enhancement of toxicity of

antitransferrin receptor antibody-pseudomonas exotoxin conjugates by adenovirus." Proc Natl Acad Sci USA **80**:4134; Scott, C., V. Goldmacher, J. Lambert, J. Jackson and G. McIntyre, 1987, "An immunotoxin composed of a monoclonal antitransferrin receptor antibody linked by a disulfide bond to the ribosome-inactivating protein gelonin: Potent in vitro and in vivo effects against human tumors." J Natl Cancer Inst. **79**(5):1163-1172; Trowbridge, I. and D. Domingo, 1981, "Anti-transferring receptor monoclonal antibody and toxin-antibody conjugates affect growth of human tumor cells." Nature **294**:171-173) linked to toxic proteins have resulted in highly cytotoxic conjugates specifically toxic to cancer cells *in vitro* and *in vivo* (Marks, A., D. Ettenson, M. Bjorn, M. Lei and R. Baumal, 1990, "Inhibition of human tumor growth by intraperitoneal immunotoxins in nude mice." Cancer Res. **50**:288-292.; Scott, C., V. Goldmacher, J. Lambert, J. Jackson and G. McIntyre, 1987, "An immunotoxin composed of a monoclonal antitransferrin receptor antibody linked by a disulfide bond to the ribosome-inactivating protein gelonin: Potent in vitro and in vivo effects against human tumors." J Natl Cancer Inst. **79**(5):1163-1172; FitzGerald, D., M. Bjorn, R. Ferris, J. Winkelhake, A. Frankel, T. Hamilton, R. Ozols, M. Willingham and I. Pastan, 1987, "Antitumor activity of an immunotoxin in a nude mouse model of human ovarian cancer." Cancer Res. **47**: 111407-1410).

Toxic ribosome inactivating proteins from plants inactivate protein synthesis by enzymatically cleaving a single N-glycosidic bond of the 28S ribosomal RNA (Endo, Y. and K. Tsurugi, 1987, "Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. RNA-N-

glycosidase activity of ricin A-chain." J Biol Chem. **262**: 8128-8130). Other cytotoxic proteins that inactive ribosomes include α-sarcin, which is produced by a fungus (Endo, T. and I. Wool, 1982, J Biol Chem. **257**: 9054-9060; Endo, Y., P. Huber and I. Wool., 1983, J Biol Chem. **258**:2662-2667.) and cloacin DF13, a plasmid encoded bacteriocin (DeGraaf, F. and P. Klaasen-Boor. , 1977, Eur J. Biochem. **73**: 107-114) both of which have ribonuclease (RNase) activity. Analogous toxic mammalian proteins have not been described, but some members of the ribonuclease superfamily in mammals have interesting, possibly related, biological properties. A human serum ribonuclease (angiogenin) was shown to abolish cell-free protein synthesis by inactivating the small ribosomal subunit of rabbit reticulocyte ribosomes (St. Clair, D., S. Rybak, J. Riordan and B. Vallee, 1987, "Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of ribosomes." Proc Natl Acad Sci USA **84**: 8330-8334; St. Clair, D., S. Rybak, J. Riordan and B. Vallee, 1988, "Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of 40S ribosomes." Biochemistry **27**:7263-7268). Cytotoxic eosinophile granule proteins also have been reported to have RNase activity (Slifman, N., D. Loegering, D. McKean and G. Gleich, 1986, "Ribonuclease activity associated with human eosinophil-derived neurotoxin and eosinophil cationic protein." J Immunol. **137**(9): 2913-2917; Gullberg, U., B. Widegren, U. Arnason, A. Egesten and I. Olsson, 1986, "The cytotoxic eosinophil cationic protein (ECP) has ribonuclease activity." Biophys Biochem Res Comm. **139**(3):1239-1242), and the sequence of human eosinophil-derived neurotoxin

(EDN) is identical to that of the nonsecretory ribonuclease from human urine (Beintema, J.J. *et al.*, 1988, Biochemistry **27**:4530-38). The present inventors have found that eosinophile proteins are also potent inhibitors of cell-free protein synthesis. In addition, antitumor (Vescia, S., D. Tramontano, G. Augusti-Tocco and G. D'Allessio, 1980, Cancer Res. **40**:3740-3744; Matousek, J., 1973, Experientia **29**:858-859) and antispermatogenic action (Dostal, J. and J. Matousek, 1973, J. Reprod. Fert. **34**:197-200) have been reported for bovine seminal ribonuclease.

SUMMARY OF THE INVENTION

The present invention stems from research of the inventors aimed at identifying mammalian proteins with potential for use as toxic moieties in cytotoxic reagents that selectively kill cells having a given surface marker. More specifically, the present invention resulted from efforts to determine whether or not mammalian RNAase, delivered to cells via the transferrin receptor-mediate endocytosis pathway, would be toxic to cells.

Thus, it is an object of the present invention to provide cytotoxic reagents that selectively kill cells having a given surface marker, comprising a toxic moiety that is a mammalian protein which is endogenous to the species in which the reagent is intended for use or is otherwise minimally immunogenic. Further, it is an object of this invention to provide toxic moieties with less systemic toxicity than presently known toxins used in directed cytotoxic reagents. In particular, it is an object of the present invention to provide direct cytotoxic reagents comprising mammalian proteins with

ribonucleolytic activity and antibodies or receptor binding ligands that recognize specific markers on tumor cells or virus-infected cells.

Bovine pancreatic RNase A is used in an initial embodiment of the present invention because of its mammalian origin and its ready availability. Further, it is known that administration of bovine RNase A to human patients (in the treatment f tick-borne encephalitis, for example; Glukhov, B. N., Jerusalimsky, A.P., Canter, V. M., and Salganik, R. I., 1976, Arch. Neurol. 38:598-603) does not produce any allergic reactions, and this seem to be connected with its weak antigenic activity. This application discloses a new use for RNAse. When it is chemically linked to a receptor-specific ligand, such as transferrin (Tfn), the complex specifically kills target cells bearing the Tfn-receptor. This particular directed toxin complex is but one convenient exemplary embodiment of the broader concept of the present invention where a mammalian protein is used as a toxic moiety in a directed cytotoxin. This complex further comprises a recognition moiety capable of specific binding with a chosen cell surface marker, where preferably both moieties are endogenous to the species in which the cytotoxin complex is to be used, thereby minimizing the immunogenicity of the complex and extending the period of useful administration.

Accordingly, the present invention relates to a selective cytotoxic reagent consisting essentially of: a toxic moiety that is a mammalian protein or a modified form thereof; a recognition moiety that binds a specific cellular surface marker; and a means of linking said toxic moiety and said recognition moiety,

wherein binding of said recognition moiety to said surface marker on a cell thereby causes said toxic moiety to kill said cell. To minimize the immunogenicity of the complex, preferably the toxic mammalian protein in this cytotoxic reagent is endogenous to the species in which the reagent is intended for use.

In the presently preferred embodiment of this cytotoxic reagent, the toxic mammalian protein has ribonucleolytic activity, for example, bovine ribonuclease A or, most preferably for human applications, human angiogenin. As noted above, other members of the ribonuclease superfamily in mammals have biological properties related to ribonuclease A and angiogenin and, therefore, are useful as the toxic moiety in the present invention, including bovine seminal ribonuclease, cytotoxic eosinophile granule proteins, and human eosinophil-derived neurotoxin (EDN) which is identical to the nonsecretory ribonuclease from human urine.

The recognition moiety of the cytotoxic reagent according to the present invention is preferably a mammalian protein or a modified form thereof. To further reduce immunogenicity, preferably the recognition moiety of the cytotoxic reagent is endogenous to the species in which said reagent is intended for use. In one of the presently preferred embodiments for human application, the recognition moiety is human transferrin or a modified form thereof, which preferentially binds cells with high levels of the transferrin receptor, for example, certain tumor cells. This embodiment is preferred for human applications in compartments where endogenous levels of transferrin are sufficiently low to avoid competitive

interference with binding of the reagent to the transferrin receptor. However, conveniently, the recognition moiety may also be an antibody or a modified form thereof which binds a specific cellular surface marker on the type of cells that are to be killed. In another embodiment exemplified herein, which is preferred for human applications where endogenous levels of transferrin are sufficiently high to cause competitive interference with binding of the reagent to the transferrin receptor, the recognition moiety is an antibody or portion thereof which recognizes the transferrin receptor in such a way that it is not competitively blocked by transferrin.

In reference to the toxic and recognition moieties, a modified form of a protein includes chemically modified forms as well as mutant forms created through genetic engineering. Chemical modifications include, for example, derivitization for the purpose of linking the moieties to each other, either directly or through a linking compound, by methods that are well known in the art of protein chemistry. In the presently preferred embodiment, the means of linking the toxic moiety and the recognition moiety comprises a heterobifunctional coupling reagent which ultimately contributes to formation of an intermolecular disulfide bond between the two moieties. Other types of coupling reagents that are useful in this capacity for the present invention are described, for example, in U.S. patent 4,545,985, Pseudomonas exotoxin conjugate immunotoxins. Alternatively, the intermolecular disulfide may conveniently be formed between cysteines in each moiety which occur naturally or are inserted by genetic

engineering. The means of linking moieties may also use thioether linkages between heterobifunctional crosslinking reagents or specific low pH cleavable crosslinkers or specific protease cleavable linkers or other cleavable or noncleavable chemical linkages. The means of linking moieties of the cytotoxic reagent may also comprise a peptidyl bond formed between moieties which are separately synthesized by standard peptide synthesis chemistry or recombinant means.

Possible chemical modifications of the protein moieties of the present invention also include derivatization with polyethylene glycol (PEG) to extend time of residence in the circulatory system and reduce immunogenicity, according to well known methods (see for examples, Lisi, P. J., Van Es, T., Abuchowski. A., *et al.*, 1982, Enzyme Therapy, Applied Biochem. 4:19-33; Beauchamp, C. O., Gonias, S. L., Menapace, D. P. *et al.*, 1982, Anal. Biochem. 131:25-33; and Goodson, R. J., and Katre, N.V., 1990, Bio/Technology 8:343-346).

Possible genetic engineering modifications of the proteins of the cytotoxic reagent include combination of the relevant functional domains of each into a single chain multi-functional biosynthetic protein expressed from a single gene derived by recombinant DNA techniques. (See, for example, application WO8809344-A, Recombinant multifunctional protein having an antibody binding site and a sequence for biological activity, ion sequestering or binding to a solid support).

Other genetic engineering modifications of the protein moieties of the cytotoxic reagent of this invention include deletions of functionally unnecessary domains to reduce the size of the protein or to modify

other parameters which facilitate production or utility, such as sequence changes to affect the solubility (e.g., cysteine to serine) or glycosylation sites. One skilled in the art would appreciate that many additional well known chemical and genetic modifications of proteins may be advantageously applied to any protein which, like the present cytotoxic reagent, is intended for parenteral administration.

The cytotoxic agent of the present invention may be directed toward various different types of cells by appropriate selection of a recognition moiety that binds to a specific cellular surface marker found specifically or predominantly on the type of cell that is to be selectively killed. For example, the cytotoxic reagent of this invention includes those with a recognition moiety that binds to a tumor cell-specific surface marker, of which many are known in the art. In the presently preferred embodiment for a human application, the recognition moiety is human transferrin or a modified form thereof, which preferentially binds cells with high levels of the transferrin receptor, particularly certain tumor cells, as noted in the Background.

Further, for selectively killing cells that have been infected with an infectious agent, the recognition moiety advantageously binds to a marker of that infectious agent on the surface of an infected cell. Of particular importance for human clinical applications *in vivo* are reagents comprising recognition moieties for cells infected by a virus, especially including latent or chronic virus infections, for examples, Epstein-Barr Virus, herpesviruses (herpes simple types I and II), hepatitis viruses (B, non-A-non-B, and delta), herpes

zoster, and cytomegalovirus. Although the recognition moiety of virus-specific cytotoxic reagents of this invention conveniently may be an antibody, advantageously the anti-viral recognition moiety may be a cellular receptor for a virus or a modified form thereof. In particular, given the known antigenic variability of the HIV-1 virus envelope protein, which limits the utility of any one antibody species in recognition of cells expressing that viral protein, for selective killing of the HIV-1 infected cells in AIDS patients, the recognition moiety of the present cytotoxic agent is advantageously the CD4 receptor protein. This receptor protein is the point of recognition between the HIV-1 virus and its specific target cell and, therefore, the envelope proteins of all immunologically distinct HIV-1 strains must nevertheless be able to bind to the CD4 protein. Thus, use of the CD4 protein in the present invention will direct the toxic moiety to kill cells infected by HIV-1 and expression on their surface the envelope protein which normally becomes embedded in the surface of cells producing that viral protein.

For treatment of patients infected with an intracellular parasite, including, for example, malaria, the recognition moiety is directed to a component of the agent which appears on the surface of infected cells, such as a marker for the merozoite form of a malaria parasite.

The present invention also relates to a pharmaceutical composition comprising a cytotoxic reagent of the present invention and a pharmaceutically acceptable carrier. Advantageously, the pharmaceutical composition is suitable for parenteral administration.

The cytotoxic reagent of the present invention may be administered by various means appropriate for different purposes, for example, for treating tumors in various parts of the body, according to methods known in the art for other immunotoxins. (See, for example, Rybak, S.M. and Youle, R.J., 1990, "Human Cancer Immunology", in Immunology and Allergy Clinics of America, W. B. Saunders, and references cited therein). These means of administration include, for example, injection by intravenous, intrathecal, intratumoral, intraocular, and intraarterial routes, to bathe particular tumor beds or the brain in high concentrations of the reagent; administration directly into the wound during surgery (intracranially, for example); administration in an enema (for colon cancer, for instance); administration in the form a spray into the bronchi or nasopharyngeal cavity; administration topically (for example, for treating melanoma); administration by drip into the peritoneal cavity (e.g., for certain forms of ovarian cancer); and administration transvaginally or transurethrally. Accordingly, the present invention also relates to pharmaceutical compositions comprising a cytotoxic reagent of this invention and a pharmaceutically acceptable carrier, particularly such compositions which are suitable for the above means of administration.

Further, the present invention relates to a method of selectively killing cells using a selective cytotoxic reagent consisting essentially of: a toxic moiety that is a mammalian protein or a modified form thereof; a recognition moiety that binds a specific cellular surface marker; and a means of linking said toxic moiety and said recognition moiety, wherein binding

of said recognition moiety to said surface marker on a cell thereby causes said toxic moiety to kill said cell. This method of the present invention may be used for cell separation *in vitro* by selectively killing unwanted types of cells, for example, in bone marrow prior to transplantation into a patient undergoing marrow ablation by radiation, for killing leukemia cells or T-cells that would cause graft-versus-host disease.

For methods of use *in vivo* , preferably the mammalian protein of the reagent used in this method is endogenous to the species in which said reagent is intended for use. Specific *in vivo* methods of this invention include a method for the chemotherapeutic alleviation of cancer in mammals comprising administering a chemotherapeutically alleviating amount of a selective cytotoxic reagent according to the present invention. This invention further comprises a method for the chemotherapeutic alleviation of an infectious disease in mammals comprising administering a chemotherapeutically alleviating amount of a selective cytotoxic reagent according to the present invention.

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples and Figures included therein.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. SDS gel analysis under reducing and non-reducing conditions of Tfn-RNase conjugate. The purified conjugate was analyzed on a 10-20% gradient SDS-acrylamide gel and compared to unreacted RNase and transferrin. The samples were reduced by boiling for 5 min. in SDS sample buffer containing 2-mercaptoethanol.

Lane 1, RNAse. Lane 2, Transferrin. Lane 3, Tfn-RNase conjugate. Lane 4 Tfn-RNase conjugate with the disulfide linkage reduced with β-mercaptoethanol.

Fig. 2. Inhibition of protein synthesis in K562 cells by Tfn-RNase conjugate compared with component proteins. K562 cells (1×10^5 cells/ml) were plated into 96 well microtiter plates and treated with varying concentrations of Tfn-RNase (filled circles), RNase (filled squares), or SPDP derivatized RNase (filled triangles). Additional sets of wells contained RNase or SPDP RNase mixed with 1×10^{-6}M human transferrin (----). Transferrin alone had no effect on protein synthesis. After 24 hours in the presence of additions, the protein synthesis rate was determined by [^{14}C]leucine incorporation. The data points are determined from the mean of triplicate incubations. The SEM was 10% or less. 100% = 7×10^3 cpm [^{14}C]leucine incorporation.

Fig. 3. Time course of protein synthesis inhibition caused by Tfn-RNase in K562 cells. K562 cells (2×10^5 cells/ml) were treated as described in the legend to Fig. 2. Different concentrations of Tfn-RNase were added and the cells were processed for [^{14}C]leucine incorporation as described. The times indicated include a 1 hour pulse with [^{14}C]leucine. The data points were determined as described in the legend to Fig. 2.

Fig. 4. Clonogenic growth assay of K562 cells treated with Tfn-RNase. K562 cells (1.4×10^5 cells/ml) were treated with buffer A, 0.1M NaCl-0.1M $NaPO_4$, pH7.2 or buffer A which contained SPDP-RNase (7×10^{-5}M). Another set in the same experiment contained K562 cells treated with buffer B, 0.1M $NaPO_4$, pH7.2 or buffer B containing Tfn-RNase at 10^{-6} or 10^{-7}M. After 24 hour treatment the

cells were washed, diluted into complete medium and plated into 96 well microtiter plates (10 wells/dilution). After 14 days the fraction of surviving cells was calculated using the Spearmen-Karber estimator (Johnson, E. and B. Brown, 1961, "The Spearman estimator for serial dilution assays." Biometrics **17**: 79-88). In this assay a 10 fold difference is considered statistically significant.

Fig. 5. Inhibition of protein synthesis by a monoclonal antibody against the Tfn receptor coupled to RNAse. Cells were incubated as described in Figure 2 with Fr27, a monoclonal antibody B3-25 against the human transferrin receptor coupled by a disulfide linkage to RNase. Fr28 is a repeat experiment. SPDP-RNase alone was also incubated with K562 cells.

DESCRIPTION OF SPECIFIC EMBODIMENTS

A presently preferred embodiment of the present invention is exemplified by a cytotoxic reagent in which the toxic moiety is bovine pancreatic ribonuclease A and the recognition moiety is human transferrin, the target being tumor cells having high levels of the transferrin receptor. RNase A is the first enzyme for which the amino acid sequence was determined and is so well defined chemically and physically that it has been a major test protein in the study of a wide variety of methods in protein chemistry since the 1950s. (Blackburn, P., and Moore, S., The Enzymes XV Part B: 317-433). Bovine RNase A is a monomeric, non-glycosylated basic protein (PI 9.5) with a M_r of 13.8 KDa, that cleaves RNA endonucleolytically to yield 3'-phospho-mono- and oligonucleotides ending in Cp or Up. Thus, it is a member of the pyrimidine-specific mammalian neutral and

alkaline RNases (reviewed in Beintema, J.J., *et al.*, 1988, Biochemistry **27**:4530-38).

Preparation of Transferrin-RNase Conjugate. A heterobifunctional reagent, N-succinimidyl 3-(2-Pyridyldithio) propionate (SPDP), was used to introduce 2-pyridyl disulfide groups into bovine pancreatic RNase. The derivatized RNase was reacted with 2-iminothiolane (2-IT) treated human transferrin (Tfn) to form a conjugate via intermolecular disulfide bond formation. RNase-Tfn conjugates were separated from unreacted RNase and Tfn multimers by gel filtration on a TSK-3000 HPLC column. Analysis of the conjugate by SDS acrylamide gel electrophoresis under reducing and non-reducing conditions showed that the conjugate had a higher MW than transferrin and upon reduction the conjugate broke apart into proteins migrating equivalent to RNase and transferrin (Fig. 1). The purified conjugate did not appear to contain significant amounts of free transferrin or RNase (Fig. 1). HPLC analysis as well as integration of the amounts of RNase and transferrin appearing upon reduction of the conjugate is consistent with the conjugate containing Tfn-RNase conjugate preparation in an approximate 1:1 molar ratio.

Intact RNase-transferrin conjugate displayed potent RNase activity that was linear in the range from 1 to 10 nM in an assay that measured release of acid soluble nucleic acids from tRNA. This activity was 3-10 times less than that expected from the calculated amount of RNase in different conjugate preparations compared to a known standard.

Inhibition of [^{14}C]Leucine Incorporation by Tfn-RNase. The RNase-Tfn conjugate inhibits protein

synthesis in several human and non-human derived cell lines. Increasing concentrations of Tfn-RNase from 10^{-9} to 10^{-7} M inhibited protein synthesis in a dose dependent manner as determined by the incorporation of [^{14}C]leucine into K562 cells (Fig. 2). The IC_{50} for different conjugate preparations and different assays ranged from $8x10^{-9}$ M to $8x10^{-8}$ M. The dose response curves for two different conjugate preparations in the same assay are shown in Fig. 2. Both dose response curves decline steeply from about 80% to 10% protein synthesis with a 10-fold increase in concentration and maximum inhibition lowers incorporation of label to 1-2% of control. Bovine pancreatic RNase or SPDP derivatized RNase only inhibits incorporation of the [^{14}C]leucine label into K562 cells at 1 x 10^{-4} M or more (Fig. 2). In experiments where complete dose response curves for RNase were obtained it required a 100 fold increase in RNase to effect the 80% to 10% decrease in protein synthesis. The SPDP treated RNase was consistently about 10-fold more potent than untreated RNase. The increased activity of the conjugate compared to RNase depends on the chemical linkage to Tfn since mixtures of Tfn and RNase do not significantly affect protein synthesis compared to RNase alone (Fig. 2). Protein synthesis in guinea pig L2C cells, monkey Vero cells and human TE671 rhabdomyosarcoma cells was also inhibited by Tfn-RNase ($5x10^{-8}$ M-$1x10^{-7}$ M).

Tfn-RNase inhibits protein synthesis at concentrations 10,000 fold lower than free RNase. To demonstrate that transferrin mediates the toxicity of the conjugate via binding the transferrin receptor, excess transferrin was incubated with the conjugate to compete for binding. The data in Table 1 shows that 70 μg/ml of

Tfn blocks the action of Tfn-RNase 10-fold. To address the role of RNase in the conjugate two inhibitors of pancreatic bovine RNase were added to the protein synthesis inhibition assay. The addition of PRI or a new more potent inhibitor of RNase to culture medium along with Tfn-RNase blocked the activity of the conjugate. Thus both protein components of the conjugate are required for the inhibition of protein synthesis by the Tfn-RNase conjugate.

Kinetics of Protein Synthesis Inhibition Caused by Tfn-RNase in K562 cells. Cytotoxic proteins such as ricin and diphtheria toxin inhibit protein synthesis following a dose dependent lag period (Olsnes, S., K. Sandvig, K. Refsnes and A. Pihl, 1976, "Rates of different steps involved in the inhibition of protein synthesis by the toxic lectins abrin and ricin." J Biol Chem. **257**:3985; Uchida, T., A. Pappenheimer and A. Harper, 1973, "Diphtheria toxin and related proteins." J Biol Chem. **248**: 3845) The inhibition of protein synthesis is first order and log linear versus time. The time course for Tfn-RNase at four different concentrations is shown in Fig. 3. Like ricin and diphtheria toxin Tfn-RNase exhibits a dose dependent lag time and then protein synthesis decreases according to a first order process. The highest concentration of Tfn-RNase ($5x10^{-7}$M) presented in Fig. 3 was saturating since higher concentrations did not significantly increase the steepness of the slope (not shown).

The rate of protein synthesis inactivation by both ricin and diphtheria toxin increases with increasing concentration of the toxin. However, the relationship between the rate of killing by the toxins and

concentration of toxin is not linear but increases proportional to the square root of the toxin concentration. This diminishes the achievable extent of killing of target cells. Advantageously, the relationship between the killing rate and the concentration of the Tfn-RNase conjugate does not follow the square root function as does ricin and diphtheria toxin but correlates linearly with concentration. This should enable increasing doses of Tfn-RNase to yield proportionate gains in log target cell kill.

The rate of intoxication of Tfn-RNase was calculated and compared to data similarly calculated from published figures for ricin A-chain conjugated with transferrin (Tfn-RTA) (Raso, V. and M. Basala, 1984, "A highly cytotoxic human transferrin-ricin A chain conjugate used to select receptor-modified cells." _J Biol Chem_. **259**:1143-1149) and one of the most 1984#9] and one of the most potent ITs reported to date, transferrin coupled to a binding deficient mutant of DT (Tfn-CRM107) (Greenfield, L., V. Johnson and R. Youle, 1987, "Mutations in diphtheria toxin separate binding from entry and amplify immunotoxin selectivity." _Science_ **238**:536-539). Measured in logs/hour, Tfn-RNase inactivated protein synthesis at 6.5 times the rate of Tfn-RTA (Table 2) and was only 1.7 times slower than Tfn-CRM107.

Clonagenic Assay of Tfn-RNase. The extent of the cell killing in clonagenic assays was performed. After 24 hours in the presence of conjugate K562 cells were washed, resuspended in complete medium, diluted serially and plated into 96 well microtiter plates. The wells that contained surviving cells were scored after 2

wks and the results of a representative experiment is presented in Fig. 4. Tfn-RNase (10^{-7}) killed between 2-3 logs of cells and a concentration of 10^{-6} M killed at least 6 logs of cells. In contrast to the results in the protein synthesis assay (Fig. 1) the clonogenic assay indicates no toxicity of 7 x 10-5 M SPDP-RNase. The elimination of 6 logs of cells can be compared to 3-4 log cell kill for RTA conjugates.

Animal Toxicity. Transferrin in plasma is about 4 mg/ml in man and may block the toxicity of Tfn-RNase in vivo. The central nervous system in an important site of metastases of peripheral tumors such as breast and lung cancer as well as a site of primary tumors. The CSF fluid contains 14 μg/ml free transferrin, an amount that would block the toxicity of Tfn-RNase only 2 fold. The toxicity of Tfn-RNase to animals was examined after direct injection into the CSF fluid. Tfn-RNase was injected into the CSF fluid of rats and guinea pigs to yield an initial concentration of 2 x 10-6 M, twice the concentration that killed 6 logs of cells in culture. No toxicity was observed in these animals.

Monoclonal Antibodies Coupled to RNase. A monoclonal antibody specific for the human transferrin receptor, B3/25 was coupled to RNase by the same method as transferrin resulting in a disulfide coupled conjugate. Incubating K562 cells for 24 hours with the conjugate, B/25-RNase at 1 x 10^{-7}M to 1 x 10^{-8} M resulted in 50 to 80% inhibition of protein synthesis (Fig. 5). The antibody is specific for the human transferrin receptor and does not bind the green monkey transferrin receptor. A green monkey cell line, Vero was not affected by the same concentration of B3/25-RNase

conjugate.

MATERIALS AND METHODS

The following materials and methods are used in the practice of the present invention.

Materials. Bovine pancreatic RNase A was purchased from CALBIOCHEM (San Diego, CA). Human placental ribonuclease inhibitor (PRI) from Promega Biotech (Madison, WI) and Inhibit-ACE RNase was from 5'-3' (Paoli, PA). Human transferrin and tRNA type X was from Sigma (St. Louis, MO). Dithiothreitol (DTT), N-Succinimidyl 3-(2-Pyridyldithio)propionate (SPDP),2-Iminothiolane (2-IT) were purchased from Pierce Chemical Co. (Rockford, IL). Plastic 96 well microtiter plates were from Nunc (Gaithersburg, MD) and all cell culture supplies were from GIBCO (Grand Island, NY).

Cell Lines. K562 (human erythroleukemia-derived cell line) was grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), 2mM glutamine, 1mM sodium pyruvate, and 10 μg/ml gentamycin. Vero (monkey kidney cell derived line), TE671 (human myosarcoma derived cell line) were maintained in Dulbecco's modified Eagle's medium with the above supplements. The cell lines were grown at 37—C in 5% CO_2 in a humidified atmosphere. L_2C leukemia is a spontaneous transplantable B cell leukemia of Strain 2 guinea pigs. L_2C leukemia cells were harvested from the blood of animals in the terminal stage of the leukemia as previously described (Zovickian, J. and R. Youle, 1988, "Efficacy of intrathecal immunotoxin therapy in an animal model of leptomeningeal neoplasia." J Neurosurg. **68**:767-774). The prepared cells were used within 24 h.

Immunotoxins. RNase was modified with SPDP as

described (Carlsson, J., H. Drevin and R. Axen., 1978, "Protein thiolation and reversible protein-protein conjugation.: Biochem. J. **173**: 723-737) and under the conditions used 1.5-mol SPDP was incorporated per mol RNase. Transferrin was reacted with 2-IT as described (Johnson, V., D. Wilson, L. Greenfield and R. Youle, 1988, "The role of diphtheria toxin receptor in cytosol translocation." J Biol Chem. **263**:1295-1300), and incubated with SPDP-RNase (RNase:Tfn 10:1 mol:mol) 18-24 h at 4—C. The conjugate was purified by gel filtration on a TSK-3000 high pressure liquid chromatography (HPLC) column. Individual peaks were characterized biologically using inhibition of protein synthesis in K562 cells as an assay. The greatest activity was associated with the peak that contained transferrin and RNase in a 1:1 molar ration which was determined by reducing the conjugate to its individual proteins followed by HPLC analysis. The amount of total protein in the conjugate was quantified by Lowry assay using BSA as a standard.

Protein Synthesis Assay. Protein synthesis in cells growing in suspension or in adherent cells was measured as previously described (Johnson, V., D. Wilson, L. Greenfield and R. Youle, 1988, "The role of diphtheria toxin receptor in cytosol translocation." J Biol Chem. **263**: 1295-1300). Briefly, cells were plated at concentrations given in Figure or Table legends into 96 well microtiter plates in leucine free RPMI 2640 medium without fetal calf serum in a volume of 100 μl. Sample or control additions were added in a volume of 10 μl and the plates were incubated at 37—C for the times indicated for each experiment. Phosphate buffered saline

containing 0.1 μCi of [^{14}C]leucine (20 μl) was added for 1 hr and the cells were harvested onto glass fiber filters using a PHD cell harvester, washed with water, dried with ethanol and counted. The results are expressed as % of [^{14}C]leucine incorporation in the mock-treated cultures. All determinations were done at least four times.

Clonagenic Cell Assay. The number of clonagenic cells surviving treatment with conjugate or other additions was determined by using a limiting dilution assay. Cells were treated with additions in a 1 ml volume in 24 well plates for 18-24 hr under the same culture conditions described in the section on protein synthesis assays. The cells were harvested by centrifugation and washed with complete culture medium. The washed cells were resuspended in complete growth medium and 6 serial 10 fold dilutions were made. Ten aliquots (100 μl) of each dilution were plated in 96-well microtiter plates. Plates were incubated for 14 days at 37—C in a humidified atmosphere. Medium was replenished every 3-4 days. Wells with growing colonies were scored by examination under an inverted phase microscope. The number of clonagenic cells remaining from the original number treated was calculated using a Spearman-Karber estimator (Johnson, E. and B. Brown, 1961, "The Spearman estimator for serial dilution assays." Biometrics **17:** 79-88).

RNase Assay. tRNA was dissolved at 1 mg/ml in water and added to a reaction mixture containing RNase and buffer (Tris, 0.5M, pH 7.5, EDTA 5 mM, human serum albumin, 0.5 mg/ml) in a total volume of 300 ml in polypropylene microfuge tubes. The mixture was incubated

for 30 min at 37—C and then placed on ice. Perchloric acid (6%, 700 ml) was added and the mixture was left on ice for 10 min. and then microfuged for 10 min. at 4—C. An aliquot of the supernatant was read at 260nm. The unknowns were compared to a standard curve of bovine pancreatic RNase A. This assay was modified from a detailed protocol described by Bond (Bond, M., 1988, "An in vitro binding assay for angiogenin using placental ribonuclease inhibitor." Anal Biochem. **173**: 166-173).

* * * * * * * * * * *

For purposes of completing the background description and present disclosure, each of the published articles, patents and patent applications heretofore identified in this specification are hereby incorporated by reference into the specification.

The foregoing invention has been described in some detail for purposes of clarity and understanding. It will also be obvious that various combinations in form and detail can be made without departing from the scope of the invention.

WHAT IS CLAIMED IS:

1. A selective cytotoxic reagent consisting essentially of:

a toxic moiety that is a mammalian protein or a modified form thereof;

a recognition moiety that binds a specific cellular surface marker; and

a means of linking said toxic moiety and said recognition moiety,

wherein binding of said recognition moiety to said surface marker on a cell thereby causes said toxic moiety to kill said cell.

2. The cytotoxic reagent according to claim 1 wherein said mammalian protein is endogenous to the species in which said reagent is intended for use.

3. The cytotoxic reagent according to claim 1 or claim 2 wherein said mammalian protein has ribonucleolytic activity.

4. The cytotoxic reagent according to claim 3 wherein said mammalian protein is bovine pancreatic ribonuclease A.

5. The cytotoxic reagent according to claim 3 wherein said mammalian protein is human angiogenin.

6. The cytotoxic reagent according to claim 1 or claim 2 wherein said recognition moiety is a mammalian protein or a modified form thereof.

7. The cytotoxic reagent according to claim 6 wherein said recognition moiety is endogenous to the species in which said reagent is intended for use.

8. The cytotoxic reagent according to claim 7 wherein said recognition moiety is human transferrin or a modified form thereof.

9. The cytotoxic reagent according to claim 6 wherein said recognition moiety is an antibody or a modified form thereof.

10. The cytotoxic reagent according to claim 6 wherein said recognition moiety binds to a tumor cell-specific surface marker.

11. The cytotoxic reagent according to claim 6 wherein said recognition moiety binds to a marker of an infectious agent on the surface of an infected cell.

12. The cytotoxic reagent according to claim 11 wherein said infectious agent is a virus.

13. The cytotoxic reagent according to claim 12 wherein said recognition moiety is a cellular receptor for a virus or a modified form thereof.

14. The cytotoxic reagent according to claim 13 wherein said receptor is the CD4 receptor for the HIV-1 virus.

15. The cytotoxic reagent according to claim 11 wherein said infectious agent is an intracellular parasite.

16. The cytotoxic reagent according to claim 1 or 2 wherein said means of linking said toxic moiety and said recognition moiety comprises a peptidyl bond.

17. The cytotoxic reagent according to claim 16 wherein said reagent consists of a single chain multi-functional biosynthetic protein expressed from a single gene derived by recombinant DNA techniques.

18. The cytotoxic reagent according to claim 1 or 2 wherein said means of linking said toxic moiety and said recognition moiety comprises a heterobifunctional coupling reagent.

19. The cytotoxic reagent according to claim 1 or 2 wherein said means of linking said toxic moiety and said recognition moiety comprises an intermolecular disulfide bond.

20. The cytotoxic reagent according to claim 1 or 2 wherein either said toxic moiety or said recognition moiety or both of said moieties are prepared by recombinant DNA or chemical peptide synthesis techniques.

21. A pharmaceutical composition comprising a cytotoxic reagent according to claim 1 or 2 and a pharmaceutically acceptable carrier.

22. A pharmaceutical composition according to claim 21, suitable for parenteral administration.

23 A method of selectively killing cells using a selective cytotoxic reagent comprising:

a toxic moiety that is a mammalian protein or a modified form thereof;

a recognition moiety that binds a specific cellular surface marker; and

a means of linking said toxic moiety and said recognition moiety,

wherein binding of said recognition moiety to said surface marker on a cell thereby causes said toxic moiety to kill said cell.

24. The method of claim 23 wherein said mammalian protein is endogenous to the species in which said reagent is intended for use.

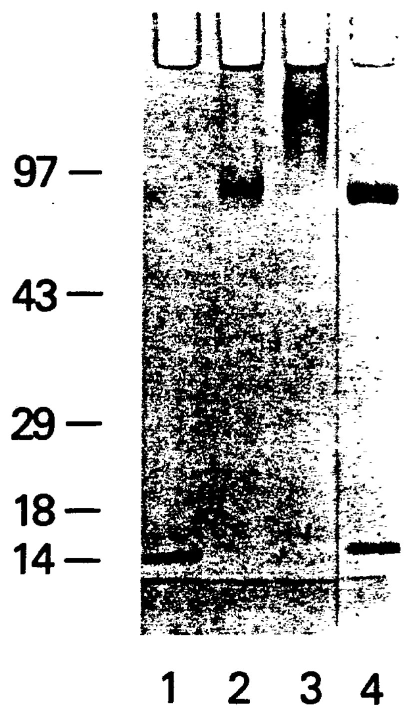

FIG. 1

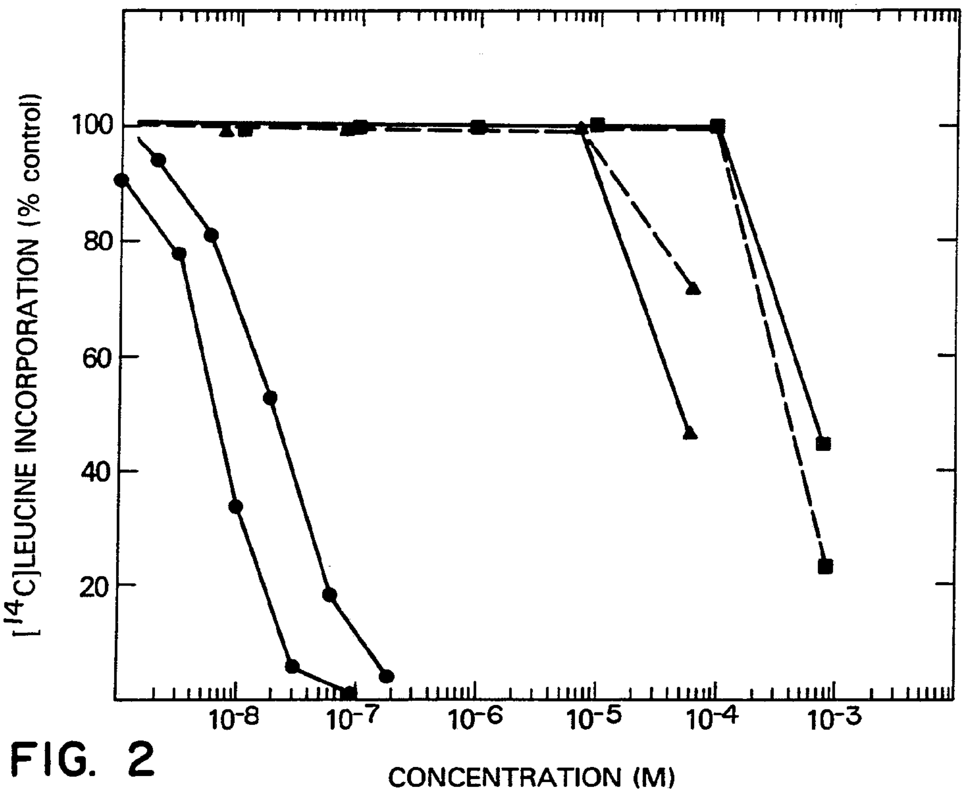
100
80
60
40
20
[14C]LEUCINE INCORPORATION (% control)
10-8
10-7
10-6
10-5
10-4
10-3
CONCENTRATION (M)

FIG. 2

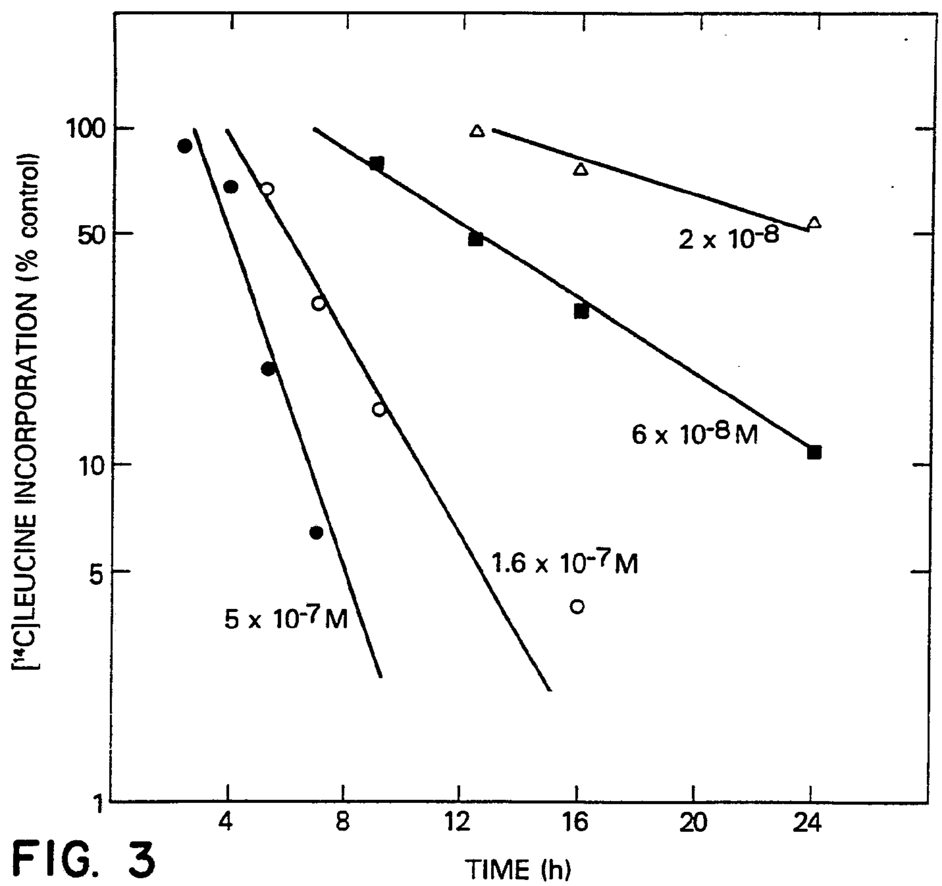
100
50
10
5
1
[14C]LEUCINE INCORPORATION (% control)
2 x 10-8
6 x 10-8M
1.6 x 10-7M
5 x 10-7M
4
8
12
16
20
24
TIME (h)

FIG. 3

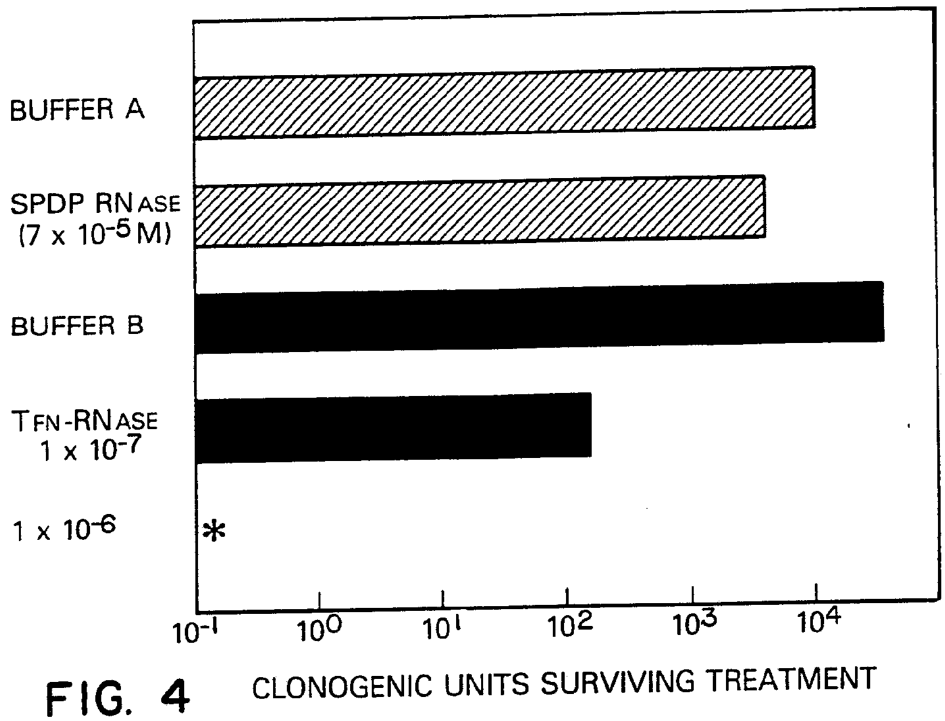
BUFFER A
SPDP RNASE
(7 x 10-5 M)
BUFFER B
TFN-RNASE
1 x 10-7
1 x 10-6
*
10-1
100
101
102
103
104
CLONOGENIC UNITS SURVIVING TREATMENT

FIG. 4

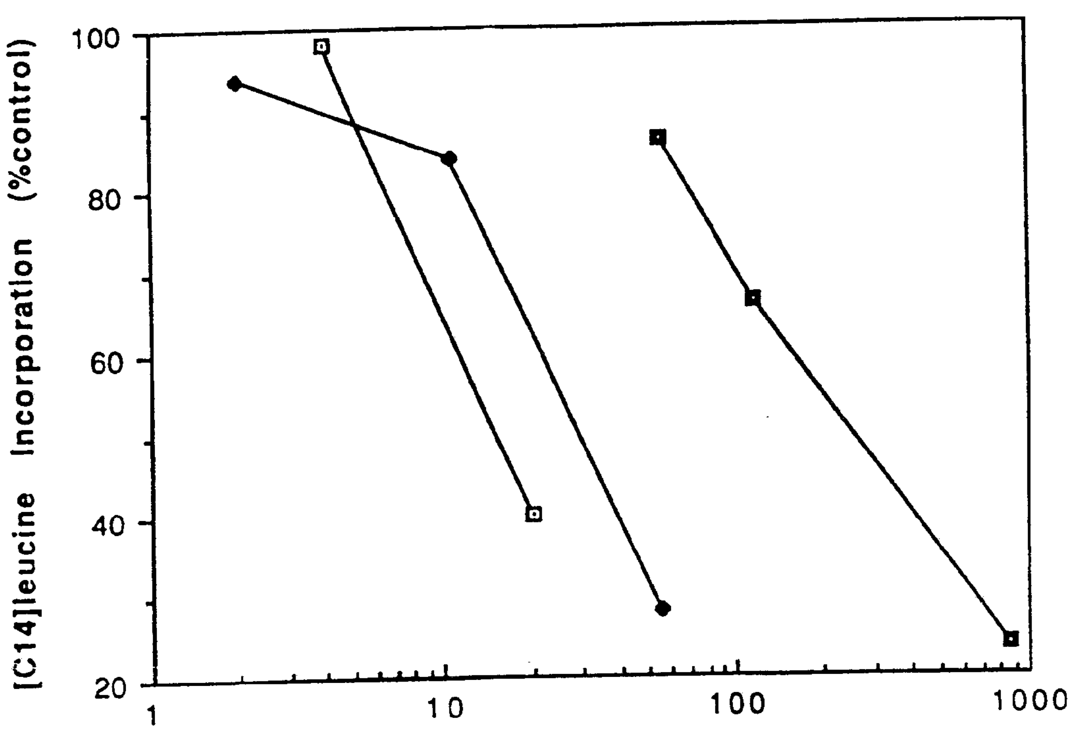
[C14]leucine Incorporation (%control)
100
80
60
40
20
1
10
100
1000
Protein Concentration (µg/ml)

FIG. 5

INTERNATIONAL SEARCH REPORT

International Applic... No. PCT?US91/01587

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(5): A61K 37/52, 39/395, 37/02; C07K 13/00
U.S.CL.: 424/85.91, 94.3, 94.5; 530/402

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	435/69.7; 935/47; 424/85.91, 94.3, 94.5; 530/402

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Proceedings of the National Academy of Sciences, vol. 77, No. 8, issued August 1980, Gilliland et al., "Antibody-directed cytotoxic agents: Use of monoclonal antibody to direct the action of toxin A chains to colorectal carcinoma cells," pages 4539-4543, see entire document.	1,6,9-24
Y	The Journal of Biological Chemistry, vol. 263, issued 25 January 1988, Johnson et al., "The Role of the Diphteria Toxin Receptor in Cytosol Translocation", pages 1295-1300, see entire document.	1-3,6-8 21-24
X / Y	Biochemical Journal, Volume 173, issued 1978, Carlsson et al., "Protein Thiolation and Reversible Protein-Protein Conjugation", pages 723-737, see entire document.	1-3,6-10 18-21 / 11-17,22-24

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 May 1991	05 JUL 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	K. Hendricks K. Hendricks

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Proceedings of the National Academy of Sciences, volume 84, issued December 1987, St. Clair et al., "Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of ribosomes", pages 8330-8334, see entire document.	1-5,23-24

FURTHER INFORMA N CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers____________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Invention I: Claims 1-4, 6-8 16-20, and 23-24, A cytotoxic reagent and method of use.
Invention II: Claim 5 a species of 1st product reagent.
Invention III: Claims 9,10 species of 1st product reagent.
See attached sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Invention IV: 11-15 species of 1st product reagent.

Invention V: 21-22 2nd product, a pharmaceutical composition.

Groups I-IV do not meet the requirement for Unity of Invention. Groups I-IV are materially distinct products each from the other and Group V is materially different from Groups I-IV.